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Sphaerodes mycoparasites and new *Fusarium* hosts for *S. mycoparasitica*

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Abstract — A comprehensive key, based on asexual stages, contact mycoparasitic structures, parasite/host relations, and host ranges, is proposed to distinguish those species of *Sphaerodes* that are biotrophic mycoparasites of *Fusarium*: *S. mycoparasitica*, *S. quadrangularis,* and *S. retispora*. This is also the first report of *S. mycoparasitica* as a biotrophic mycoparasite on *Fusarium culmorum* and *F. equiseti* in addition to its other reported hosts (*F. avenaceum*, *F. graminearum,* and *F. oxysporum*). In slide culture assays, *S. mycoparasitica* acted as a contact mycoparasite of *F. culmorum,* and *F. equiseti* producing hook-like attachment structures. Fluorescent and confocal laser scanning microscopy showed that *S. mycoparasitica* is an intracellular mycoparasite of *F. equiseti* but not of *F. culmorum.* All three mycoparasitic *Sphaerodes* species were observed to produce asexual (anamorphic) stages when challenged with *Fusarium*. Furthermore, a phylogenetic tree, based on (large subunit) LSU rDNA sequences, depicted closer relatedness to one another of these *Fusarium*-specific *Sphaerodes* taxa than to the non-mycoparasitic *S. compressa, S. fimicola,* and *S. singaporensi*s.

Key words — ascomycete, coevolution

Introduction

Mycoparasitism refers to the parasitic interactions between one fungus (parasite) and another fungus (host). These relationships can be categorized as either necrotrophic or biotrophic (Boosalis 1964; Butler 1957). Differences between necrotrophic and biotrophic mycoparasites were reviewed and outlined by Jeffries & Young (1994). This paper emphasizes biotrophic *Sphaerodes* Clem. (*Ascomycota*) mycoparasites and their association with fungi, in particular *Fusarium* Link. Biotrophic mycoparasitic ascomycete and basidiomycete fungi are characterized by intimate contact with host cells (Bauer & Oberwinkler 2004; Gams et al. 2004), with or without penetration. This intimate contact involves generation of short haustoria and appressoria or absorptive mycoparasitic cells.

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The cytoplasm of the host hyphae remains healthy in at least some phase(s) of mycoparasitic interactions (Jeffries 1995).

Among pyrenomycetous orders, *Melanosporales* contains the largest number of biotrophic mycoparasites (Davey et al. 2008; Zhang et al. 2002), mainly within *Melanospora* Corda, *Persiciospora* P.F. Cannon & D. Hawksw., *Sphaerodes,* and *Syspastospora* P.F. Cannon & D. Hawksw. (Cannon & Hawksworth 1982; Harveson & Kimbrough 2001; Posada et al. 2004). *Sphaerodes* is a relatively small genus with unique morphological features to some extent similar to *Melanospora* and *Microthecium* Corda (García et al. 2004). Interestingly, most of the known *Sphaerodes* mycoparasitic taxa associate with *Fusarium* species — causal agents of diseases in plants and toxicosis in humans and animals (Goh & Vujanovic 2010; Harveson & Kimbrough 2001; Vujanovic & Goh 2009). To distinguish *Sphaerodes* from other genera in *Melanosporales*, ascospore characters such as wall ornamentation and shape are utilized (Zhang et al. 2002).

Identification of *Sphaerodes* species is mostly based on morphological attributes of their ascomata, structural details of ascomatal wall and neck tissues, as well as distinctive ascospore shape and ornamentation. To date, their anamorphs and their mode of mycoparasitism of *Fusarium* are poorly known.

Among all the described *Sphaerodes* species, five have been reported associated with fungal hosts (Farr & Rossman 2009). *Sphaerodes mycoparasitica* Vujan., *S. quadrangularis* Dania García, Stchigel & Guarro and *S. retispora* (Udagawa & Cain) P.F. Cannon & D. Hawksw. var. *retispora* were reported to be biotrophic mycoparasites of *Fusarium* species (Vujanovic & Goh 2009; Goh & Vujanovic 2010; Harveson & Kimbrough 2001), whereas *S. episphaeria* (W. Phillips & Plowr.) Clem. was associated with *Hypomyces* sp. (Cannon et al. 1985). *Sphaerodes retispora* var. *retispora* was the first *Sphaerodes* species reported to be a biotrophic mycoparasite of *Fusarium oxysporum* (Harveson & Kimbrough 2001). Recently, *S. mycoparasitica* and *S. quadrangularis* were also observed to establish biotrophic mycoparasitic relationships with a few *Fusarium* taxa, including red-pigmented species such as *F. avenaceum* and *F. graminearum* (Goh & Vujanovic 2010; Vujanovic & Goh 2009). However, there is no single report comparing these three *Sphaerodes* biotrophic mycoparasites, specific to *Fusarium,* in terms of differences in mycoparasitic contact structures, host ranges, and anamorphic reproductive structures.

Therefore, the purpose of this paper is to document two new *Fusarium* hosts for *S. mycoparasitica*, as well as to discuss and describe differences in these three biotrophic mycoparasites based on parasitic contact structures, phialidic stages and host ranges. Furthermore, a phylogenetic analysis based on LSU (large subunit) rDNA is incorporated into this study to determine the role of host specialization in the evolution of mycoparasitic *Sphaerodes*.

Materials and methods

Fungal isolates and growth

Sphaerodes mycoparasitica was first isolated and described by Vujanovic & Goh (2009) as an obligate biotrophic mycoparasite of various *Fusarium* taxa from Canadian agricultural fields. *Sphaerodes quadrangularis* (CBS112764 strain) was first reported as a facultative biotrophic mycoparasite of *Fusarium avenaceum*. *Sphaerodes retispora* var. *retispora* (CBS 994.72), isolated from Japanese soil, was also obtained from Centraalbureau voor Schimmelcultures (CBS, Fungal Biodiversity Centre) Baarn, The Netherlands. Biotrophic mycoparasite *Sphaerodes mycoparasitica* SMCD2220 and pathogenic *Fusarium* strains (*F. arthrosporioides* SMCD2247*, F. culmorum* SMCD2248*, F. equiseti* SMCD2134*, F. flocciferum* SMCD2135*, F. poae* SMCD2136*,* and *F. torulosum* SMCD2139) were obtained from the Saskatchewan Microbial Collection and Database (SMCD), Saskatchewan, Canada. All fungal isolates were grown and maintained on potato dextrose agar (PDA) (Difco, BD, Sparks, Maryland) prior to the study.

Fungal-fungal interactions

For examination of the interaction between isolates of *Sphaerodes* and *Fusarium* species, both biotrophic mycoparasite and *Fusarium* isolates were inoculated and assessed using slide culture assays proposed in Cole & Kendrick (1968) and Jacobs et al. (2005), with slight modifications as in Goh & Vujanovic (2010). Slides were maintained in a sterile humidity chamber as outlined in Kavková & Čurn (2005) and daily observations on the hyphal interactions at the meeting place (contact zone) were performed under a Carl Zeiss Axioskop2 equipped with Carl Zeiss AxioCam ICc1 camera with 20×, 40× and 100× objectives. Formation of biotrophic mycoparasitic contact structures attaching *Sphaerodes* species to *Fusarium* hyphae were examined, recorded, and compared to drawings from the literature (Jordan & Barnett 1978; Rakvidhyasastra & Butler 1973; Whaley & Barnett 1963). Diameters of both parasitized and non-parasitized *Fusarium* hyphal cells were measured under light microscopy with a 100× objective lens. Each treatment used six replications consisted of *Sphaerodes* or *Fusarium* alone, and *Sphaerodes–Fusarium* co-inoculated. The experiment was repeated twice. In the slide-culture assay, *Fusarium* mycelia infected with *Sphaerodes* haustoria were stained with lactofuchsin (Carmichael 1955). Stained hyphae of both *Fusarium* and *Sphaerodes* in slide-culture were then examined with a Carl Zeiss Axioskop2 fluorescent microscope attached to Carl Zeiss AxioCam ICc1 with 40× and 100× objectives. Slide-culture assays were also subjected to Zeiss META 510 confocal laser scanning microscopy (CLSM) analysis to observe intracellular mycoparasitism under a C-Apochromat 63× N.A.1.2 phase-contrast water immersion objective through Z-stacking mode to scan through the *Fusarium* hyphae with intracellular infection (CLSM with 514nm excitation – argon and LP585 emission filters) (Abdellatif et al. 2009).

Fungal morphology and taxonomy

The anamorphic stages of three mycoparasitic *Sphaerodes* species (*S. mycoparasitica, S. quadrangularis,* and *S. retispora* var. *retispora*) were compared in the presence of *Fusarium* hosts. Diameters of base and neck of monophialides were measured and base-

neck ratios were calculated. Genomic DNA of *S. retispora* var. *retispora* CBS 994.72 was extracted, amplified, and sequenced as outlined in Vujanovic & Goh (2009) by targeting LSU rDNA fragments with LS1/LR5 primers (Hausner et al. 1993; Rehner & Samuels 1995; Zhang & Blackwell 2002). The LSU sequence from this study and sequences retrieved from GenBank were aligned using Clustal X software (version 1.82) (Thompson et al. 1997), and edited in BioEdit (Hall 1999). Distance trees were generated with Phylogenetic Analysis Using Parsimony (PAUP) 4.0b10 software (Swofford 2000) using neighbor-joining approach, and validated using bootstrap analyses with 1000 repetitions. A fungal distance tree was prepared with sequences showing bootstrap values higher than 50%. The LSU sequence from *Sphaerodes retispora* var. *retispora* was submitted to GenBank as GU205261.

Statistical analysis

The difference in diameters of parasitized and non-parasitized hyphal cells was analyzed with a T-test (SPSS 1990).

Results

Fungal-fungal interaction

Hyphae-hyphae interactions and contact structures in the contact zone were examined for seven days. On day three, *Sphaerodes mycoparasitica* was found to produce hook-shaped contact structures on *Fusarium equiseti* and *F. culmorum* (Fig. 1). On day five, more hook-shaped contact structures and intracellular penetration of *F. equiseti* were observed (Fig. 2A, 3A–D). The combination of lactofuchsin dye and fluorescent or confocal laser scanning microscopy revealed that the parasitized or penetrated *Fusarium* cells became empty (loss of cytoplasm = no fluorescence) or fluoresced with low intensity (very pale) (Fig. 3A–D) as compared to healthy *Fusarium* cells. During the seven days of observation, no *S. mycoparasitica* hyphae were observed within *F. culmorum* cells. *Sphaerodes mycoparasitica* produced hook-shaped contact structures (Fig. 1A, a) more frequently than clamp-like contact structures (Fig. 1B, b) on both *F. equiseti* and *F. culmorum.* Diameters of *F. equiseti*, but not *F. culmorum*, hyphae parasitized by *S. mycoparasitica* were observed to be significantly reduced compared to non-parasitized *Fusarium* hyphae (with T-test, $P = 0.001$ and $P > 0.05$, respectively) (FIG. 4).

None of the *Fusarium* taxa tested appeared to be suitable hosts for mycoparasitic *S. quadrangularis* and *S. retispora,* even after 10 days of co-inoculation on slide cultures. No contact biotrophic parasitic structures or intracellular parasitism by *S. quadrangularis* and *S. retispora* on the tested *Fusarium* strains were observed at the interaction or contact zone. Also, *F.* arthrosporioides*, F. flocciferum, F. poae,* and *F. torulosum* did not appear to be suitable hosts for *S. mycoparasitica.* Around five days after inoculation

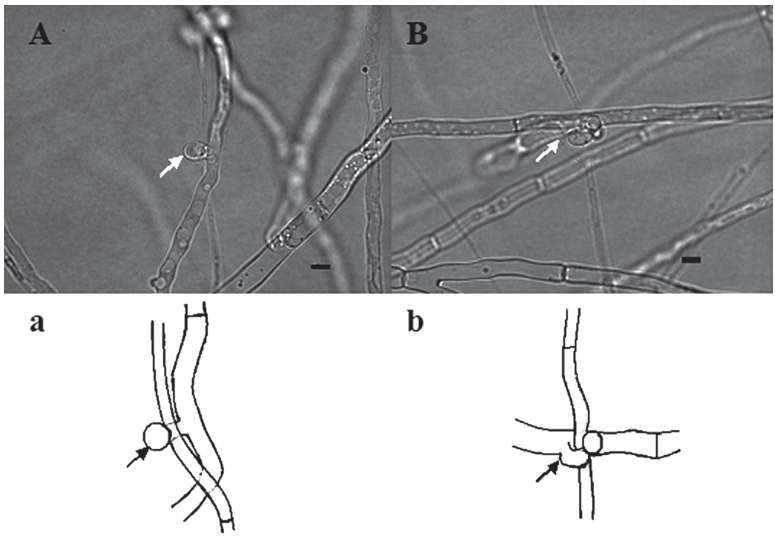

Fig. 1. *Sphaerodes mycoparasitica–Fusarium* spp. mycoparasitism assays. (A–a). Hook-shaped contact structures (arrows). (B–b). Clamp-like clasping cells (arrows). Figures a and b are diagrammatic drawings for both A and B. Scale bars = 5μm.

on slide culture assays, mycelia of *F.* arthrosporioides were inhibited by *S. mycoparasitica*. *Fusarium* arthrosporioides started to form rosette-like mycelia at the contact zone with *S. mycoparasitica* (Fig. 2B).

On the fifth and seventh days after inoculation, anamorphic structures were produced by *S. mycoparasitica* more abundantly in the zone of contact with *F. culmorum* (Fig. 2C, D). Anamorphic structures or asexual organs in close proximity to *F. culmorum* mycelia were red-colored (Fig. 2D), whereas the organs at a distance were not (Fig. 2C).

Fungus-fungus coevolution

Six *Sphaerodes* and one *Melanospora* species — *S. compressa* (Udagawa & Cain) P.F. Cannon & D. Hawksw., *S. fimicola* (E.C. Hansen) P.F. Cannon & D. Hawksw., *S. mycoparasitica, S. quadrangularis, S. retispora* var. *retispora, S. singaporensis* (Morinaga, Minoura & Udagawa) Dania García, Stchigel & Guarro, *Melanospora brevirostris* — were phylogenetically analysed. Information related to these strains is summarized in Table 1. Node M_1 is the point of divergence between the three *Fusarium*-specific *Sphaerodes* spp. and the other four taxa (Fig. 5; Table 1).

The phylogenetic tree further shows that the three *Sphaerodes* mycoparasites of *Fusarium* species — *S. mycoparasitica, S. quadrangularis* and *S. retispora* —

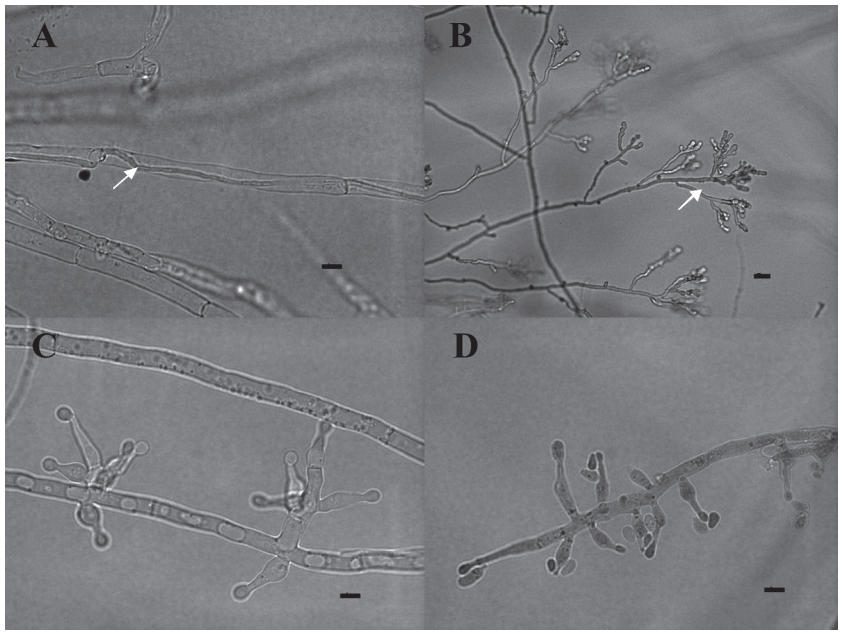

Fig. 2. Intracellular parasitism, hyphal inhibition response, and anamorphic stages during the *Sphaerodes mycoparasitica–Fusarium* spp. interactions. (A). Intracellular parasitism by *S. mycoparasitica* in *F. equiseti* (arrow). (B). *Fusarium* hyphal inhibition response when challenged with *S. mycoparasitica*; deformation of hyphae into rosette-like shapes (arrow). (C). Hyaline *S. mycoparasitica* anamorphic stages. (D). *Sphaerodes mycoparasitica* anamorphic stages with adsorption of red pigments from *F. culmorum*. Scale bars A, C, D = 5μm; B = 20μm.

diverge at M_2 to distinguish hyperparasites on white-pigmented *F. oxysporum* (such as *S. retispora*) from those on a red-pigmented *F. avenaceum* host (such as *S. quadrangularis*). Moreover, evolution from M_2 occurs at M_3 giving rise to mycoparasites of white- and red-pigmented *Fusarium*. This is the case of *S. mycoparasitica*, which attacks *F. avenaceum, F. culmorum*, *F. equiseti*, *F. graminearum*, and *F. oxysporum,* (Fig. 5; Table 1). Thus, M_3 is the point where polyspecificity as opposed to monospecificity on *Fusarium* appears.

Discussion

The small knobs or hook-shaped contact structures formed by *Sphaerodes mycoparasitica* on *Fusarium culmorum* and *F. equiseti* were similar to those described by Whaley & Barnett (1963) for *Gonatobotrys simplex* Corda [= *Melanospora damnosa* (Sacc.) Lindau] on *Alternaria tenuis* Nees [*A. alternata*], and by Jordan & Barnett (1978) for *Melanospora zamiae* Corda on *Tritirachium*

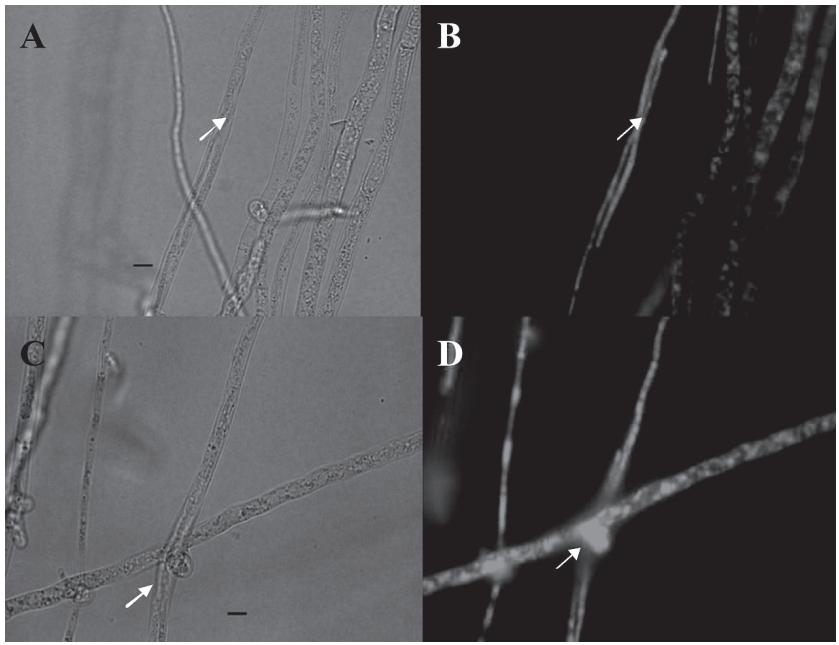

Fig. 3. (A–B) Intracellular parasitism by *Sphaerodes* inside *Fusarium equiseti* (arrows). (B–D) Intracellular hyphae produced by *Sphaerodes* inside *F. equiseti* with hook-shaped contact structure (arrows). A and C were captured under light microscopy; whereas in B and D hyphae were stained with lactofuchsin and images were captured under fluorescent and confocal laser microscopy, respectively. Scale bars = 5μm.

sp. Hook-shaped contact structures are well-known among biotrophic mycoparasites in the *Melanosporales*. Harveson & Kimbrough (2001) were the first to report *S. retispora* var. *retispora* as a contact biotrophic mycoparasite on *F. oxysporum* with hook-like contact structures. Harveson & Kimbrough (2001) also reported another melanosporaceous fungus, *Persiciospora moreaui* P.F. Cannon & D. Hawksw., as a contact biotrophic mycoparasite of *F. oxysporum* with similar contact branches as in *M. zamiae* and *S. retispora* (Harveson & Kimbrough 2000). Recently, *S. mycoparasitica* was found to produce similar hook-shaped contact structures on *Fusarium avenaceum, F. graminearum,* and *F. oxysporum* (Vujanovic & Goh 2009) and *S. quadrangularis* on *F. avenaceum* species (Goh & Vujanovic 2010). In this study, *S. mycoparasitica* was observed to form clamp- or clasp-like contact branches to attach to *F. equiseti* and *F. culmorum* (Fig. 1B, b). These structures were also reported for *Stephanoma phaeosporum* E.E. Butler & McCain, another biotrophic mycoparasite

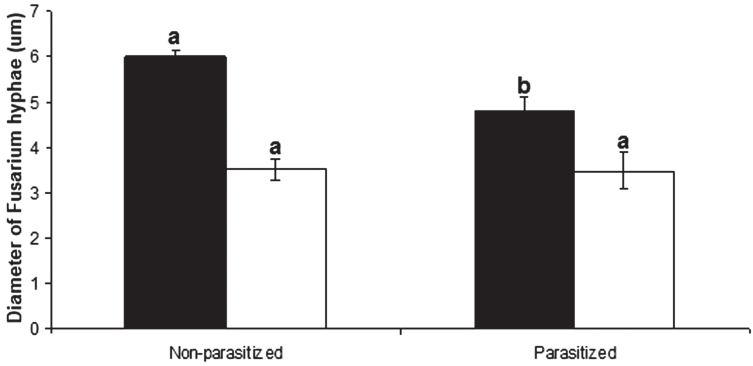

Fig. 4. Mean hyphal diameters of parasitized and non-parasitized *Fusarium equiseti* cells (■) and *F. culmorum* (□) on 1-week slide-cultures with *Sphaerodes mycoparasitica* biotrophic mycoparasite. Data are means and standard deviations. Same lowercase letters indicate no significant difference between parasitized and non-parasitized hyphae at $P = 0.05$, with T-test.

(Rakvidhyasastra & Butler 1973). These contact structures may also be employed by contact or fusion biotrophic mycoparasites as tools to acquire nutrients from the hosts (Carmichael 1955; Gams et al. 2004; Whaley & Barnett 1963). Nutrients, growth factors, biotins, mycotrophein, and thiamine have been found to be important for nourishment and proliferation of biotrophic mycoparasites (Hwang et al. 1985; Jordan & Barnett 1978).

In this study, loss of cytoplasm (Fig. 3A, C) and a reduction of the diameter of *F. equiseti* hyphae resulted from mycoparasitism (Fig. 4). Similarly, Harveson & Kimbrough (2001) noticed that *Sphaerodes retispora* and *M. zamiae* isolates reduced the total hyphal weight and aerial hyphae of *F. oxysporum,* in addition to inhibiting the growth of this *Fusarium* species. Furthermore, loss or decreased intensity of staining or colour of dye in host cells (compared to healthy) were further reported by White & Traquair (2006) as an indication of loss of cytoplasm and intracellular infection. Intracellular parasitic activity was also described in *Fusarium–Rhizoctonia* and *Mucor–Rhizopus* mycoparasitic interactions (Arora & Dwivedi 1980; Gupta & Tandon 1978; Gupta et al. 1979). Although hyphal diameter of *F. culmorum* was not reduced by *S. mycoparasitica* (Fig. 4), this could be due to the lack of intracellular penetration in *F. culmorum* during the tested period. Barnett (1963), Jordan & Barnett (1978), Jeffries & Young (1994) and Jeffries (1995), have all pointed out that biotrophic mycoparasites, in general, have narrow host ranges. Therefore, it is not surprising that not all the *Fusarium* taxa tested could act as hosts for *S. mycoparasitica, S. quadrangularis* and *S. retispora.*

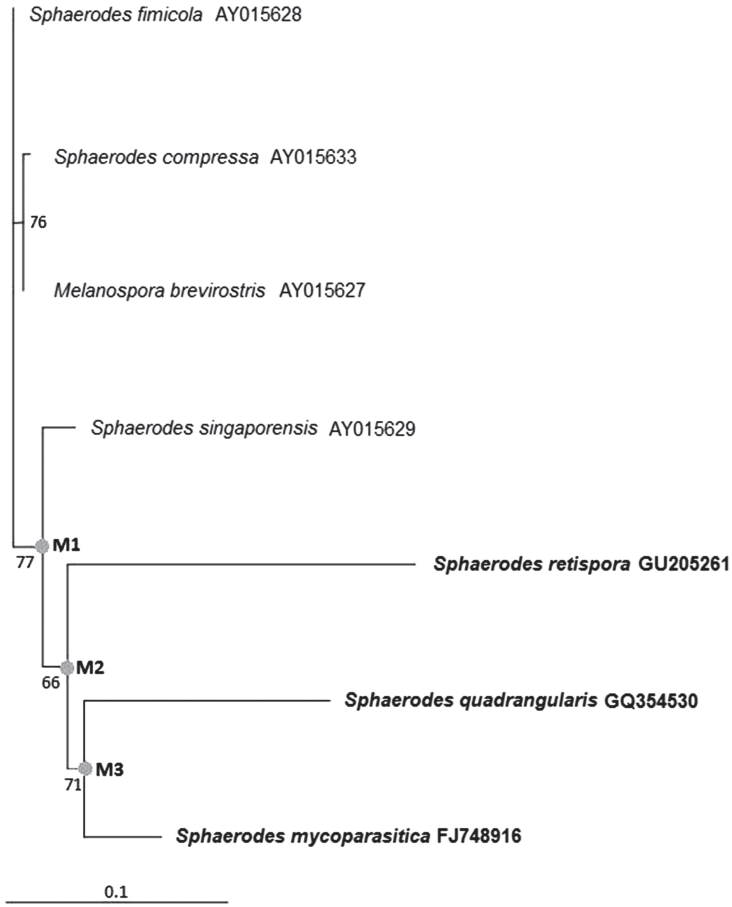

Fig. 5. Phylogenetic tree based on LSU rDNA sequences for six *Sphaerodes* species showing position of mycoparasitic taxa associated with *Fusarium* hosts. M1 – the point of evolutionary divergence between *Sphaerodes* mycoparasites associated with *Fusarium* and *Sphaerodes* taxa including closely related *Melanospora brevirostris* associated with other fungal and plant hosts; M_2 – point of branching towards specialization or monospecificity of *S. retispora* on *F. oxysporum* (white mycelium) and monospecificity of *S. quadrangularis* on *F. avenaceum* (red mycelium); M_3 – the point of evolutionary direction towards polyspecificity of *S. mycoparasitica* on various white and red *Fusarium* hosts. Bootstrap values of 50% or greater from 1000 bootstrap replications are indicated for the corresponding branches.

Table 1. Information related to six *Sphaerodes* species and a *Melanospora* species used for phylogenetic analysis.

	Isolated from	Myco-parasite	Distribution	Reference
*Melanospora brevirostris**	Dead plant stems and decaying truffles as well as on various *Pezizales*, usually *Sepultaria* sp.	Yes	England, North Europe	Cannon et al. 1985; Cannon & Hawksworth 1982; Farr & Rossman 2009
Sphaerodes compressa	Soil, cow dung, dead leaves, aerial contaminant	No	Canada, USA, Japan, New Caledonia	Cannon et al. 1985; Farr & Rossman 2009
S. fimicola	Dung, surface litter and soil, plants	No	Europe, USA, Madeira, British Isles	Cannon et al. 1985; Farr & Rossman 2009
S. mycoparasitica	Several *Fusarium* species	Yes	Canada	Vujanovic & Goh 2009
S. quadrangularis	*F. avenaceum*	Yes	Spain	García et al. 2004; Goh & Vujanovic 2010
S. retispora var. *retispora*	*F. oxysporum*	Yes	Japan, New Guinea, USA	Cannon et al. 1985; Harveson & Kimbrough 2001
S. singaporensis	Soil	Unknown	Singapore	Morinaga et al. 1978

*Note: Information on Melanospora brevirostris (Fuckel) Höhn. was also included since the LSU rDNA sequences analyses in Fig 5. of this article suggest relatedness to *S. compressa* and *S. fimicola* in concordance with findings of Davey et al. (2008).

Sphaerodes quadrangularis was first described by García et al. (2004). At the time its anamorph was unknown. Here, *S. quadrangularis* was observed to produce mono- and polyphialides or asexual organs like those of *S. mycoparasitica* (Fig. 2C) and *S. retispora* (Harveson & Kimbrough 2001) when inoculated together with *Fusarium avenaceum*. Based on *S. mycoparasitica* analyses, Vujanovic & Goh (2009) proposed that most anamorphic traits in *Sphaerodes* (e.g., hyaline and ampulliform phialides as well as irregularly branched conidiophores) resemble those of *Trichoderma* species (sect. *Pachybasium*) in *Hypocreales*. In contrast, the base-to-neck ratios of phialides in *S. mycoparasitica, S. quadrangularis*, and *S. retispora* show interspecies differences summarized in the key to taxa of *Sphaerodes*.

Key to the mycoparasitic taxa of *Sphaerodes*

1 Clamp-like contact structures present 2

1* Clamp-like contact structures lacking. Phialides with base-neck width ratio < 2; mono- and polyphialidic anamorphic stages; monospecific on *F. oxysporum* . . . . . ***S. retispora* var. *retispora***

2 Intracellular penetration and haustoria present. Phialides with base-neck width ratio between 2–2.5; mono- and polyphialidic anamorphic stages; polyspecific on *F. avenaceum, F. culmorum, F. equiseti, F. graminearum*, and *F. oxysporum* . . . . . ***S. mycoparasitica***

2* Intracellular penetration and haustoria absent. Phialides with base-neck width ratio > 2.5; mono- and polyphialidic anamorphic stages; monospecific on *F. avenaceum* . . . . . ***S. quadrangularis***

In addition, this study showed that when *S. mycoparasitica* and *F. culmorum* were co-inoculated in slide culture, anamorphic structures and hyphae of the former were red-colored (Fig. 2D). Similarly, *S. quadrangularis* hyaline hyphae became red-colored after contacting *F. avenaceum* hyphae (Goh & Vujanovic 2010). This could be due to the absorption of *Fusarium* red pigments by *Sphaerodes* through biotrophic mycoparasitism (Goh & Vujanovic 2010). However, the mechanism of this phenomenon remains unclear. The red pigments of *F. avenaceum*, *F. culmorum,* and *F. graminearum* are aurofusarin toxins (Malz et al. 2005). Perhaps host toxins drive the evolution of mycoparasites. Thus, it would be interesting for further studies, as indicated by relatedness of these *Fusarium*-specific *Sphaerodes* taxa (FIG 5.), to explore whether it is actually the nature of fusaria toxins that create an evolutionary pressure inducing specialization within *Sphaerodes*.

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